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The M3 muscarinic receptor i3 domain confers oxidative stress protection on calcium regulation in transfected COS-7 cells

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Summary

Evidence suggests that muscarinic receptors (MAChRs) are involved in various aspects of neuronal and vascular functioning, and that there is selective oxidative stress sensitivity (OSS) among MAChR subtypes. COS-7 cells transfected with M1, M2 and M4 subtypes show greater OSS than the M1 and M3 subtypes, as seen by the decreased ability of cells to extrude or sequester calcium (Ca^{2+}) following exposure to dopamine (DA) or Aβ 25–35, and depolarization by oxotremorine. We sought to determine which receptor domain may be responsible for the differential vulnerability to OS between 'OS-sensitive' (M1) and 'non-sensitive' (M3) subtypes. Comparison of the amino acid sequences of each receptor has shown that the third cytoplasmic loop (i3 loop) is the domain with the most variability between the two subtypes. Therefore, mutations were made by either deleting or exchanging the i3 loop of M1 and M3 receptors. Experiments revealed that deletions of the i3 loop increased DA sensitivity (a lower percentage of cells showing recovery of $[Ca^{2+}]_i$ following depolarization) in both receptors. Chimerics of M1 in which the i3 loop of the M3 was exchanged with the i3 loop of the M1 (M1M3i3) showed that DA sensitivity was reduced (a greater percentage of cells showing increases in calcium clearance) following depolarization. The M3 chimerics containing the M1 i3 loop (M3M1i3) offered no protection against DA-induced decrements in calcium buffering. Results suggest that the longer i3 loop of the M3 may decrease OSS, possibly playing a role in targeting antioxidants to specific receptor sites that impart OSS.

Key words: calcium regulation; chimeric; COS-7 cells; dopamine; muscarinic receptor; oxidative stress.

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Introduction

In several previous experiments we (Cutler *et al*., 1994) and others (Ferrari-DiLeo *et al*., 1995; Flynn *et al*., 1995; Claus *et al*., 1997; Ladner & Lee, 1999; Muma *et al*., 2003) have shown that there is a loss of sensitivity in muscarinic receptors (MAChRs) as a function of Alzheimer's disease (AD), as well as aging (see Joseph *et al*., 2001 for review). This sensitivity may result from alterations in receptor-G protein coupling/uncoupling (e.g. Cutler *et al*., 1994; Muma *et al*., 2003), an index that has been shown to be extremely vulnerable to oxidative stress (OS) (e.g. Venters *et al*., 1997). The implications of these findings could be important, as it is well known that MAChRs are found in memory control areas (Levey, 1996) and the vasculature (Elhusseiny *et al*., 1999; Nakai & Maeda, 2000), thus increasing their vulnerability to OS. More recent evidence suggests that muscarinic receptor activation provides protection against possible OS and subsequent apoptosis (De Sarno *et al*., 2003). Additionally, Fawcett *et al*. (2002) showed that a low-molecular-weight endogenous inhibitor from AD brain decreases oxotremorine-M binding (see also Frey *et al*., 1996). In this respect, MAChRs are involved in various aspects of both neuronal (Rossner *et al*., 1998) and vascular functioning (Elhusseiny *et al*., 1999).

Because these receptors have a variety of functions and, in particular, functions that involve their antioxidant/anti-apoptotic properties, it could be postulated that their functional declines (e.g. loss of agonist sensitivity) could have profound consequences for the aged organism. Additionally, OS vulnerability, as assessed via agonist (oxotremorine) stimulation of MAChR enhancement of dopamine (DA) release in superfused striatal slices, increases as a function of age (Joseph *et al*., 1996), resulting in significant decreases in oxotremorine stimulation of DA release.

Moreover, it appears that the OS sensitivity (OSS) among various MAChR subtypes may not be uniform. Recent findings have indicated that COS-7 cells transfected with one of the five MAChRs and exposed to DA (Joseph *et al*., 2002) showed differences in OSS expressed as a function of Ca^{2+} buffering (i.e. the ability to extrude or sequester Ca^{2+} following oxotremorine-induced depolarization). The loss of Ca^{2+} buffering in these experiments is similar to that reported in many studies with respect to aging (see Herman *et al*., 1998; Toescu & Verkhratsky, 2000). Such losses can have a substantial effect on the functioning and viability of the cell (Lynch & Dawson, 1994; Mattson *et al*., 2000; Vannucci *et al*., 2001), further increasing OS (De Sarno *et al*., 2003) and leading ultimately to decrements in motor and memory function in senescent rats (Huidobro *et al*.,

1993; Shukitt-Hale *et al*., 1998). It is also important to note that there are significant differences in the rates of aging among various brain regions, with areas such as the hippocampus (Nyakas *et al*., 1997; Kaufmann *et al*., 2001), cerebellum (Hartmann *et al*., 1996; Kaufmann *et al*., 2001) and striatum (Joseph *et al*., 1996; Kaasinen *et al*., 2000) showing profound alterations in aging in such parameters as morphology, electrophysiology and receptor sensitivity.

Given these considerations, it became of interest in the present study to determine the structural locus that imparts increased OSS in the MAChR. Because studies (Wu *et al*., 1997, 1998, 2000) have indicated that G protein βγ (a) interacts with the i3 loop of the MAChR, (b) is inhibited by the G protein α subunit, and (c) is necessary for phosphorylation and subsequent signal transduction, the i3 loop was chosen for these determinations. Interestingly, it also appears from our previous findings (Joseph *et al*., 2002; Joseph & Fisher, 2003) that the MAChRs conferring the most OSS to the transfected COS-7 cells (i.e. M1, M2, M4) had shorter i3 loops than the M3 or M5 MAChRs, suggesting that the length of the i3 loop might be involved in regulation of OSS. Thus, the present experiments were carried out to determine whether (a) deletions of the entire i3 loop in a 'non-OS-sensitive' (M3AChR) and an 'OS-sensitive' receptor (M1AChR), or (b) exchanging i3 loops between these receptors, would alter Ca^{2+} flux (reflected in baseline, peak and recovery measurements) following depolarization with oxytremorine and exposure to DA in COS-7 cells transfected with either of these mutated receptors.

DA was utilized to induce OS in these cells because it has been found to form several reactive oxygen species (ROS) agents, including: (a) oxygen-derived free radical semiquinones and quinononones (see Stokes *et al*., 1999; LaVoie & Hastings, 1999; Rabinovic *et al*., 2000); (b) reactive products of reduced oxygen, e.g. superoxide anions or OH (Fornstedt *et al*., 1990); and (c) H_2O_2 (Hastings *et al*., 1996).

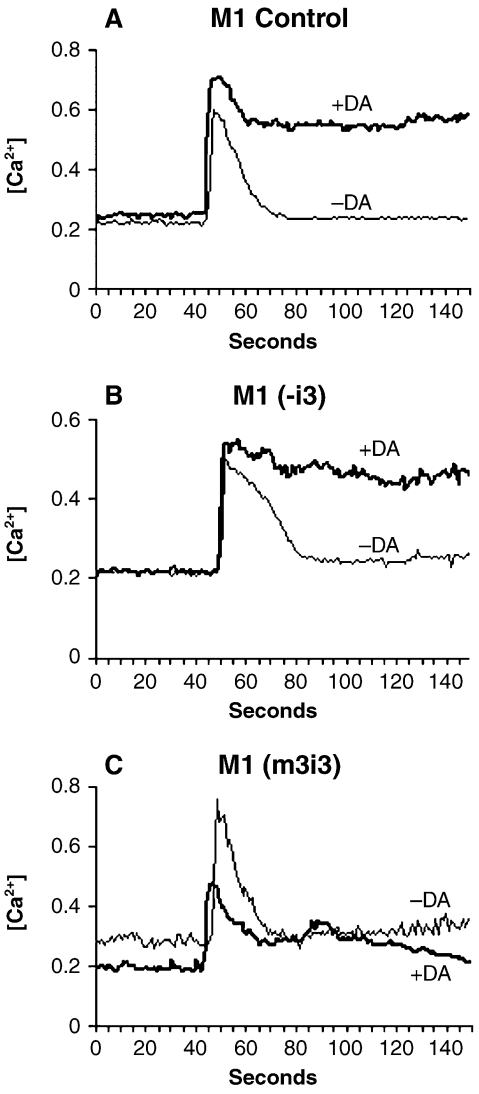


Fig. 1 (A–C) Typical tracings of $[Ca^{2+}]_i$ showing baseline, response to depolarization and recovery in COS-7 cells transfected with the M1 receptor (A) or its chimerics, M1-i3 (B) or M1M3i3 (C), with or without DA pretreatment (see Experimental procedures).

Results

Ca^{2+} imaging

Typical tracings of baseline intracellular calcium concentration ($[Ca^{2+}]_i$), increase in response to depolarization by oxytremorine, and recovery (as defined in the Experimental procedures) in COS-7 cells transfected with M1 and M3 receptors or their chimerics (e.g. M1-i3, M1M3i3) with or without DA pretreatment are shown in Figs. 1 (M1, A,B,C, respectively) and 2 (M3, A,B,C, respectively). DA was effective in decreasing Ca^{2+} buffering in the M1 wild-type, and in the M1-i3 transfected COS-7 cells (Fig. 1), but this effect was less in the M1M3i3 transfected cells. For M3, the removal of the i3 loop decreased Ca^{2+} buffering and the M3M1i3 chimeric responded only slightly differently from the M3-i3 cells (Fig. 2). The wild-type M3 was not affected by DA exposure. Note that Figs 1 and 2 show example single tracings and do not in every case reflect the findings when the entire data set was examined as described below.

As shown in Figs 3(A) and 4(A), DA exposure raised the baseline $[Ca^{2+}]_i$ levels in the COS-7 cells transfected with M1AChR wild-type ($P < 0.01$, control vs. DA treated) but not those transfected with the M3AChR wild-type or any of the mutations ($P > 0.05$, all comparisons).

Analysis of the percentage increase in $[Ca^{2+}]_i$ after oxotremorine-induced depolarization suggested that the cells transfected with the M1 or M3 wild-type receptors and exposed to DA showed no differences from their respective controls (Figs 3B and 4B, respectively). Conversely, DA-treated cells transfected with M1-i3 (Fig. 3B) or M3-i3 (Fig. 4B), respectively, showed higher increases in $[Ca^{2+}]_i$ than their respective controls ($P < 0.0001$ for both comparisons). DA also enhanced Ca^{2+} increases in cells transfected with M3M1i3 ($P < 0.0001$; Fig. 4B) but not in M1M3i3 cells ($P > 0.05$; Fig. 3B).

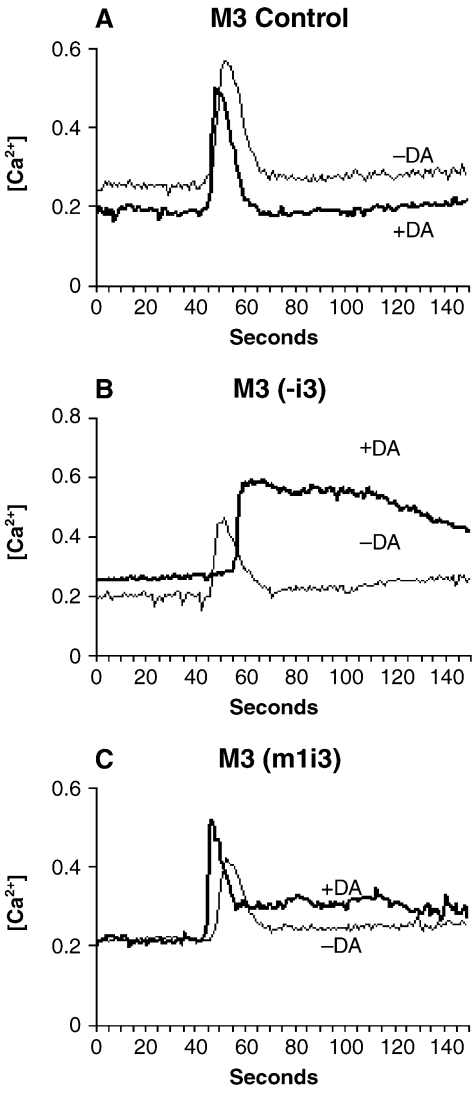


Fig. 2 (A–C) Typical tracings of $[Ca^{2+}]_i$ showing baseline, response to depolarization, and recovery in COS-7 cells transfected with M3 receptor (A) or its chimerics, M3-i3 (B) or M3M1i3 (C), with or without DA pretreatment (see Experimental procedures).

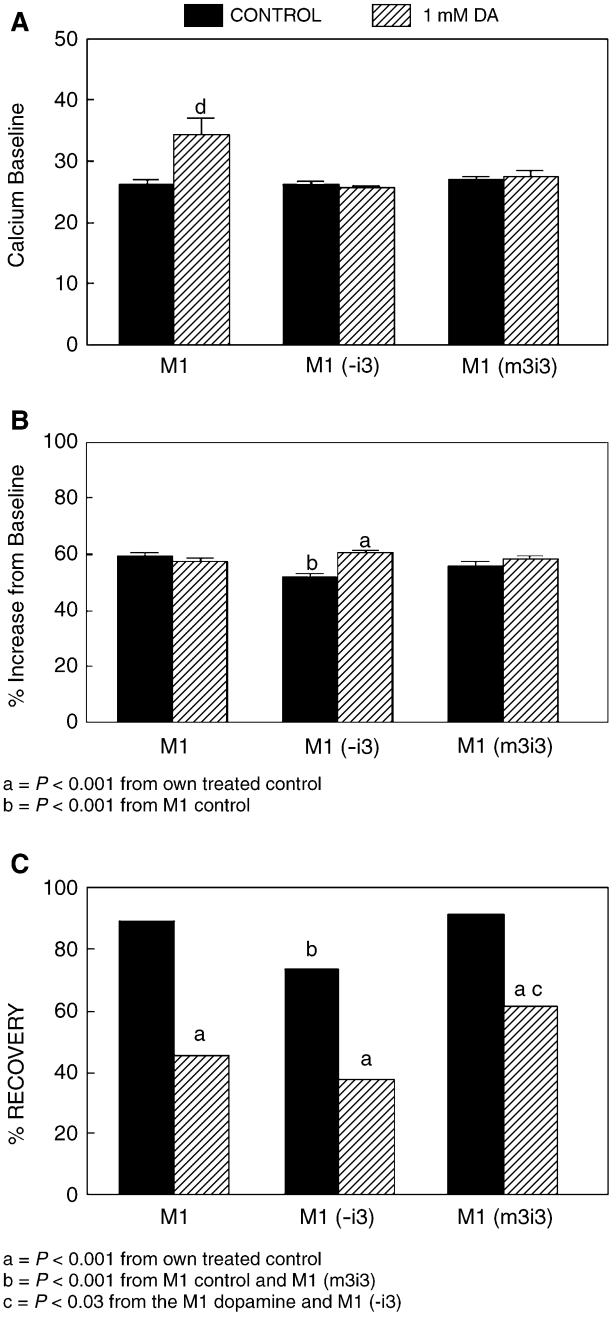


Fig. 3 (A–C) Baseline $[Ca^{2+}]_i$ (A), increase (B) and recovery (C) in COS-7 cells transfected with the M1 Receptor or its chimerics M1-i3 or M1M3i3 (see Experimental procedures). The number of cells analysed for each group was: control = 131, DA-treated = 136, M1-i3 = 114, M1-i3 DA = 171, M1M3i3 = 81, M1M3i3 DA = 78. The overall Kruskal-Wallis test statistic for recovery was 137.116 ($P < 0.000$, d.f. = 5). Individual comparisons are shown in the figures.

Differences in recovery were assessed following DA pretreatment and oxotremorine-induced depolarization of wild-type M1- and M3-transfected COS-7 cells (Fig. 3C (M1) and Fig. 4C (M3)). As can be seen, recovery was significantly reduced after DA in the M1 but not the M3 wild-type transfected COS-7 cells ($P < 0.0001$ and $P < 0.08$, respectively). Figure 3(C) also shows that there was a significant decrease in recovery in cells transfected with M1-i3 as compared with those transfected with the wild-type M1 ($P < 0.0001$) in the absence of DA. However, exposure to DA of the same M1 wild-type and M1-i3 cells showed similar decreases in recovery, both of which were significantly less than their respective controls ($P < 0.001$ for both comparisons).

A comparison of the control and DA-treated cells transfected with the M1M3i3 chimeric (Fig. 3C) showed that there was significant decline in recovery ($P < 0.001$). However, the DA-treated M1M3i3 chimeric showed greater recovery than similar cells transfected with the M1 wild-type or the M1-i3 mutation ($P < 0.025$, $P < 0.001$, respectively), suggesting that the i3 loop affords some protection of Ca^{2+} buffering after DA exposure. Additionally, the control M1M3i3 cells showed improved recovery compared with those transfected with the M1-i3.

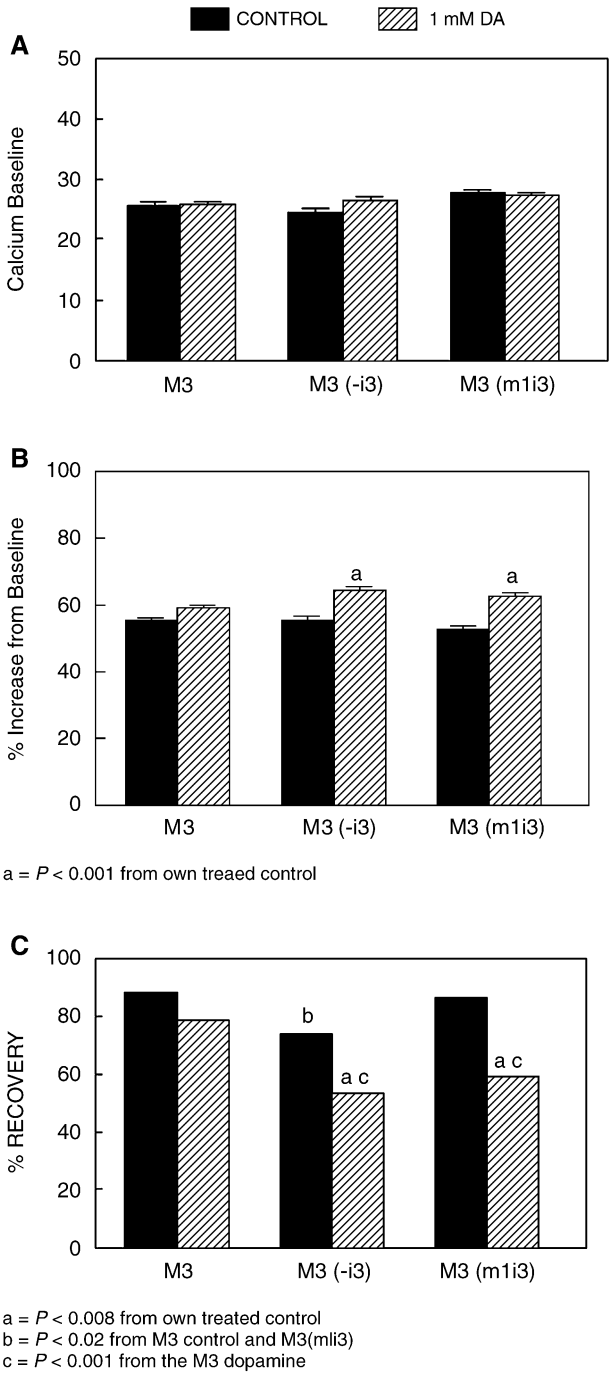


Fig. 4 (A–C) Baseline $[Ca^{2+}]_i$ (A), increase (B) and recovery (C) in COS-7 cells transfected with M3 receptor or its chimerics M3-i3 or M3M1i3 (see Experimental procedures). The number of cells analysed for each group was: control = 84, DA-treated = 136, M1-i3 = 114, M1-i3 DA = 171, M1M3i3 = 81, M1M3i3 DA = 78. Because an overall ANOVA was run for both M1 and M3 under all conditions, the *F*-value is the same as that in the legend to Fig. 3. *Post hoc* analyses for comparisons among the groups are given in the figures. The overall Kruskal-Wallis test statistic = 46.883 ($P < 0.000$, d.f. = 5). Individual comparisons were carried out by Mann-Whitney *U*-tests and are given in C.

In the case of the M3-i3 transfected cells, the results indicated that in the presence of DA this deletion significantly decreased recovery as compared with cells transfected with the wild-type M3 ($P < 0.02$). However, recovery was unaffected in the absence of DA in M3M1i3-transfected cells (M1 wild-type vs. M3M1i3, $P > 0.05$; Fig. 4C).

In the presence of DA, the M3M1i3 cells showed a significant decrease in recovery as compared with their untreated controls (non-DA exposed M3M1i3 vs. DA-exposed M3M1i3, $P < 0.001$). The decrease in recovery was similar to that seen in DA-treated compared with control M3-i3 transfected cells ($P > 0.05$) and significantly lower than that seen in M3 wild-type transfected COS-7 cells ($P < 0.001$; Fig. 4C). Typical tracings for each of these conditions are shown in Figs 1 and 2.

Discussion

The present findings showed that there are differences in the extent of recovery of $[Ca^{2+}]_i$ following depolarization and DA treatment (recovery – see Experimental procedures) between wild-type, M1- and M3AChR-transfected COS-7 cells. M1-transfected cells showed decreases in this parameter whereas the M3-transfected cells showed no effect. These findings support those of previous studies with DA (Joseph *et al.*, 2002; Joseph & Fisher, 2003) in that its effect on recovery is selective for the M1 but not the M3 muscarinic receptor subtype. As illustrated previously (Joseph & Fisher, 2003), it does not appear that the second messenger pathway is involved in OSS, because M1 and M3AChR signalling pathways are both IP_3-mediated.

Additionally, it was also apparent in the present and previous studies (Joseph *et al.*, 2002; Joseph & Fisher, 2003) that possible differences in oxotremorine-induced Ca^{2+} flux in the absence of OS could not account for the increased OS vulnerability in M1-transfected COS-7 cells, because recovery in M1- and M3-transfected cells was similar when they were not exposed to DA. These findings suggest that the locus of the increased sensitivity in the M1 receptors may possibly lie within the structure of the receptor. In this respect, we have shown previously (Joseph *et al.*, 2002) that the differences in recovery following depolarization did not appear to be dependent upon $[Ca^{2+}]_i$ in DA-pretreated cells. In this study, M2-transfected cells did not show enhanced $[Ca^{2+}]_i$ following exposure to DA greater than that seen in the control M2-transfected cells. However, the DA-treated, M2-transfected cells were not able to buffer $[Ca^{2+}]_i$ (i.e. show recovery), whereas the DA-treated, M5-transfected cells were similar to untreated controls in this regard. Thus, initial Ca^{2+} influx following depolarization does not predict the ability to buffer Ca^{2+} with oxidative stress.

Similarly, the initial increase in $[Ca^{2+}]_i$ following depolarization was not a good predictor of Ca^{2+} buffering capacity following depolarization and DA treatment in cells transfected with the M1 wild-type, M1-i3 deletion or the chimerics M1M3i3. Thus, the increase in $[Ca^{2+}]_i$ following depolarization was similar in the M1- and M1M3i3-transfected cells, but the Ca^{2+} buffering

Table 1 Amino acids, muscarinic receptor subtype and oxidative stress

	M1	M2	M3	M4	M5
His	2	6	9	6	8
Lys	24	31	38	25	38
Tyr	16	16	18	15	22

was greater in cells transfected with the M1M3i3 chimerics. Additionally, the increase in $[Ca^{2+}]_i$ was higher in the M1-i3 cells than in cells transfected with the wild-type M1, while Ca^{2+} buffering did not differ between the cells transfected with the wild-type or the M1-i3. However, in the cells transfected with the various forms of the M3 subtypes, it appeared that Ca^{2+} buffering capacity might affect recovery, because Ca^{2+} influx was higher in both the M3-i3- and the M3M1i3-transfected cells than in the wild-type, and the ability to buffer Ca^{2+} in the DA-treated cells was compromised.

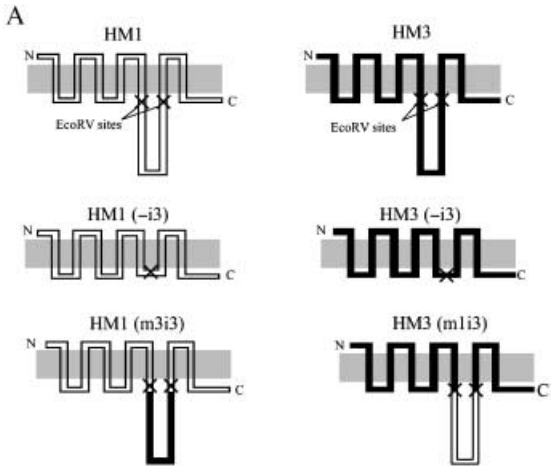
Research has indicated that the i3 loop subserves a multiplicity of functions involving signalling, phosphorylation (Wu *et al*., 1997, 1998, 2000) and even receptor internalization via arrestin binding (Lee *et al*., 2000). Thus, we believed that this structural element might be involved in conferring OS vulnerability to the receptor and, ultimately, to the transfected cells. Deletion of the entire loop appeared to increase the sensitivity to DA exposure in M3AChR, because transfection of the cells with M3-i3 receptors resulted in recovery of Ca^{2+} buffering following DA exposure that was significantly less than that seen with the wild-type M3-transfected cells, suggesting that the M3i3 loop may confer some protection against DA-induced OS. Some support for this contention was provided by the demonstration that cells transfected with M1M3i3 chimerics exhibited decreased sensitivity (as indicated by increased recovery) to DA exposure as compared with that seen in cells transfected with the M1 wild-type or the M1-i3 receptors.

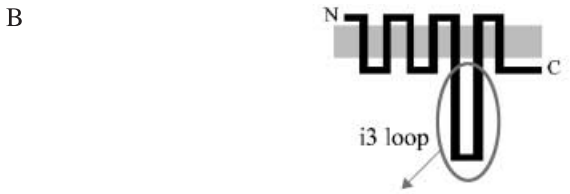
It appears, however, that the M1 i3 loop may have mixed properties, with respect to OS vulnerability. This loop appears to confer some level of protection in the M1AChR, because COS-7 cells transfected with M1-i3 receptors showed decreased recovery of Ca^{2+} buffering even in the absence of DA, as compared with those transfected with the wild-type M1 receptor. However, in the environment of the M1AChR, the i3 loop is less effective in protecting against OS than the M3 i3 loop, because cells transfected with the M3M1i3 receptors showed DA-induced deficits in recovery that were equivalent to those seen in the cells transfected with the M3-i3 and less than those seen in cells transfected with the wild-type receptors. Although we have not investigated whether the i3 loops of other OS-sensitive (M2 and M4) and OS-insensitive receptors (M5) have similar properties compared with those of the M1 and M3 i3 loops, it is clear from these findings that if the structural properties of this loop do not provide sufficient OS protection to the receptor, then function may be compromised. Thus, in addition to the range of functions alluded to above, this loop may also be responsible for providing protection against oxidative stress. Therefore, if this loop imparts OSS to the receptor, and if it is damaged via ROS, then this may contribute to the functional neuronal losses seen in aging. As shown in Table 1, for the entire receptor, the highest numbers of his, lys and tyr are found in the MAChR subtypes that show the least vulnerability to OS (His > 7, Lys > 32, Tyr > 17). The higher number of his, lys and tyr residues may impart protection through metal suspension (Table 1). Thus, M1-, M2- and M4-transfected COS-7 cells should show greater sensitivity to OS than those transfected with M3 or M5 receptor subtypes.

In addition to these considerations, one factor that could be important with respect to protection may be the length of the i3 loop, which is longer in the M3AChR. The i3 loop of the M1AChR contains 157 amino acids, whereas the M3AChR i3 loop contains 240 amino acids. It could be expected from these findings that there would be an overall higher number of all amino acids in the i3 loop, but it appears that only selected amino acids (Phe, Leu, Ser, Gln, Arg, Thr, Lys, Ala, Asp) are increased. Others were similar in number in both i3 loops (e.g. Arg, Glu, Gly). Note that the Lys component seems to be greater in both the whole receptor and the i3 loop of the M3 receptor than the M1. Perhaps the abundance of the positively charged Lys residues may be important as one locus of OS vulnerability. We plan to examine this further using site-directed mutagenesis to examine the role of Lys. Moreover, we are now analysing the peptide sequences that might contribute to the differential protection offered by these two i3 loops. As more information is gained it may be possible to carry out selective mutations to localize specific sites of vulnerability within these loops.

Questions of selective downstream OS-induced alterations in G proteins remain to be addressed. For example, Fawcett *et al*. (2002) have shown that a low-molecular-weight inhibitor isolated from AD brains inactivates MAChR by altering agonist binding. It is also important to know whether this selective sensitivity of MAChR to OS has a secondary effect on the membrane lipid microenvironment. Nevertheless, given the factors cited above, the protection of this loop via specifically targeted antioxidants, or possibly combinations of antioxidants that might be available through diets high in fruits and vegetables, may be of extreme importance in protecting these and other homologous receptors. Studies have shown, for example, that flavonoids such as myricetin and quercetin can prevent the deleterious effects of the low-molecular-weight inhibitor cited above (Fawcett *et al*., 2002), and it has been reported that diets high in antioxidant flavonoids may reduce the incidence of dementia (Commenges *et al*., 2000). Finally, findings from our laboratory (Joseph *et al*. in press) have shown that prior exposure of COS-7 cells transfected with M1AChR to extracts of berry-fruits (e.g. blueberries, cranberries) reduced sensitivity to the deleterious effects of amyloid beta treatment on Ca^{2+} buffering.

One other important consideration is that if Ca^{2+} dysregulation coupled with OS is responsible for functional changes seen in the aging nervous system then it may be that regional differences in these changes may depend upon local variations in the level of expression of various receptor subtypes. For example, Hersch



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                *** *****  *** ** * *             *                     *
HM3             RIYKETEKRTKELAGLQASGTEAETENFVHPTGSSRSCSSYELQQQSMKRSNRRKYGRCH
HM3 EcoRV       RIYKETDIRTKELAGLQASGTEAETKNFVHFTGSSRSCSSYELQQQSMKRSNRRKYGRCH
HM3 (-i3)       RIYKETD-------------------------------------------------------
HM1 (mli3)      RIYKETDIRARELAALQGSETPGKGGGSSSSSERSQPGAEGSPETPPGRCCRCCRAPRLL
HM1             RIYRETENRARELAALQGSETPGKGGGSSSSSERSQPGAEGSPETPPGRCCRCCRAPRLL
HM1 EcoRV       RIYRETDIRARELAALQGSETPGKGGGSSSSSERSQPGAEGSPETPPGRCCRCCRAPRLL
HM1 (-i3)       RIYRETD-------------------------------------------------------
HM1 (m3i3)      RIYRETDIRTKELAGLQASGTEAETKNFVHFTGSSRSCSSYELQQQSMKRSNRRKYGRCH

                     *      *       **
HM3             FWFTTKSWKPSSEQMDQDHSSSDSWNNNDAAASLKNSASSDEEDIGSETRAIYSIVLKLP
HM3 EcoRV       FWFTTKSWKPSSEQMDQDHSSSDSWNNNDAAASLENSASSDEEDIGSETRAIYSIVLKLP
HM3 (-i3)       -------------------------------------------------------------
HM3 (mli3)      QAYSWKEEEEEDEGSMESLTSSEGEEPGSEVVIKMPMVDPEAQAPTKQPPRSS-------
HM1             QAYSWKEEEEEDEGSMESLTSSEGEEPGSEVVIKMPMVDPEAQAPTKQPPRSS-------
HM1 EcoRV       QAYSWKEEEEEDEGSMESLTSSEGEEPGSEVVIKMPMVDPEAQAPTKQPPRSS-------
HM1 (-i3)       -------------------------------------------------------------
HM1 (m3i3)      FWFTTKSWKPSSEQMDQDHSSSDSWNNNDAAASLKNSASSDEEDIGSETRAIYSIVLKLP

HM3             GHSTILNSTKLPSSDNLQVPEEELQMVDLKRKADKLQAQKSVDDGGSFPKSFSKLPIQLE
HM3 EcoRV       GHSTILNSTKLPSSDNLQVPEEELQMVDLKRKADKLQAQKSVDDGGSFPKSFSKLPIQLE
HM3 (-i3)       -------------------------------------------------------------
HM3 (mli3)      -------------------------------------------------------------
HM1             -------------------------------------------------------------
HM1 EcoRV       -------------------------------------------------------------
HM1 (-i3)       -------------------------------------------------------------
HM1 (m3i3)      GHSTILNSTKLPSSDNLQVPEEELQMVDLKRKADKLQAQKSVDDGGSFPKSFSKLPIQLE

                                        *    *          *   *** *********  *
HM3             SAVDTAKTSDVNSSVGKSTATLPLSFKEATLAKR-FALKTRSQITKRKRMSLVKEKKAAQT
HM3 EcoRV       SAVDTAKTSDVNSSVGKSTATLPLSFKEATLAKR-FALKTRSQITKRKRMSLVKDIKAAQT
HM3 (-i3)       ------------------------------------------------------IKAAQT
HM3 (mli3)      -----------------PNTVKRPTKKGRDRAGKGQKPRGKEQLAKRKTFSLVKDIKAAQT
HM1             -----------------PNTVKRPTKKGRDRAGKGQKPRGKEQLAKRKTFSLVKEKKAART
HM1 EcoRV       -----------------PNTVKRPTKKGRDRAGKGQKPRGKEQLAKRKTFSLVKDIKAART
HM1 (-i3)       ------------------------------------------------------IKAART
HM1 (m3i3)      SAVDTAKTSDVNSSVGKSTATLPLSFKEATLAKR-FALKTRSQITKRKRMSLVKDIKAART
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Fig. 5 (A) Structures of the HM1 and HM3 muscarinic receptors and the respective mutations made in each. The location of the incorporated *Eco*RV sites at the amino terminus-N, and carboxyl terminus-C of the i3 loop are shown (top panels, A), and the resulting chimerics made of each by either removal of the i3 loop [HM1(- i3); HM3(- i3) (middle panels, A)], or translocation of the i3 loop [HM1(m3i3); HM3(m1i3), bottom panels (A)]. (B) Amino acid sequences of the HM1 (bold) and HM3 (italic) i3 loops and their respective mutations. Shaded regions indicate the incorporation of the *Eco*RV Site. An asterisk () indicates sequence homology between HM1(m3i3) and HM3(m1i3).

et al. (1994) showed that in the striatum, M2 receptors may be the predominant muscarinic receptor, whereas the M1 and M4 receptors are expressed in 78% and 44% of striatal neurons, respectively. In addition, Levey (1996) has shown a wide distribution of these receptor subtypes in the dentate gyrus, whereas M2 and M4 MAChR receptor proteins were found in high concentrations in the fimbria-fornix. Flynn & Mash (1993) have shown that M1 receptors were found to be enriched in the primate forebrain. As seen in the present experiment, these receptor subtypes show increased vulnerability to OS and are

found in high concentrations in brain areas that also show considerable morphological decline in aging and neurodegenerative disease. For example, Rodriguez-Puertas *et al*. (1997) showed that M1 and M2AChR were significantly decreased in entorhinal cortex and hippocampus. However, there were also declines of M3AChR in these areas but not in the striatum and neocortex. Thus, to some extent, this pattern of loss follows the pattern of localized vulnerability in oxidative stress.

Experimental procedures

Deletions and reinsertion of the i3 loop

Deletion mutations were made to the M1 or M3 receptors by excising the entire i3 loop (M1-i3 and M3-i3) (Fig. 5A, middle panels). A second mutation then involved inserting the i3 loop of one receptor (M1 or M3) by ligating it into the corresponding region of the other (M1m3i3, M3m1i3) (Fig. 5A, bottom panels). To facilitate cloning, *Eco*RV restriction sites not found in either of the M1 or M3 receptor were engineered to both ends of the i3 loop using the QuickChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Oligos containing the recombinant sequence were designed and ordered from Operon (Valencia, CA, USA). Confirmation of the mutations was performed by restriction digestion with *Eco*RV and gel analysis. The resulting digestions were extracted from the gel and purified using the StrataPrep DNA gel extraction kit (Stratagene). The resulting fragments were used to construct the M1m3i3 and M3m1i3 chimerics. The deletion mutations and chimerics were then transformed, purified as above, transfected into the COS-7 cells and tested as described below.

Sequences

Confirmation of the mutations was achieved by ds-sequencing by Certigen (Lubbock, TX, USA), and by restriction digestion (Fig. 5B).

Cell culture, transfections, and treatments

COS-7 cells

COS-7 cells (ATCC) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and containing 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin sulphate. Twenty-four hours prior to transfection, cells were harvested with trypsin, counted and plated on 100-mm^2 tissue culture plates at 5×10^6 cells per plate. Cells were transiently transfected with rat muscarinic receptor subtype 1 or 3 DNA (Bonner *et al*., 1987, 1988) or their chimerics by the DEAE-dextran method. After transfection, cells were incubated for 2.5 h in growth medium containing 80 μM chloroquine to minimize degradation of the DNA. Transfected cells were then maintained in growth medium for 48 h, harvested with trypsin, plated onto coverslips in 35-mm plates and incubated overnight.

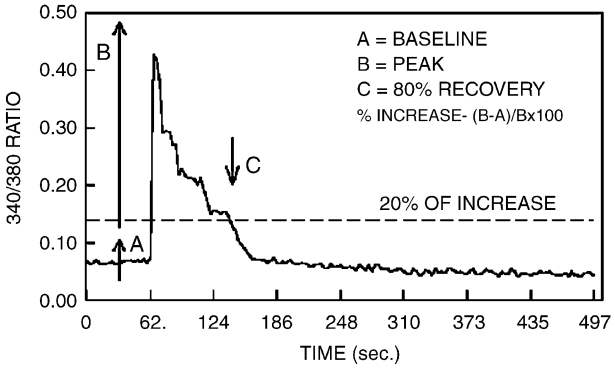

Fig. 6 Typical image analysis response ratios for $[Ca^{2+}]_i$ (340/380 ratio) in control cells transfected with MAChR (see Experimental procedures). Cellular $[Ca^{2+}]_i$ was analysed using three parameters: (A) baseline, (B) increase and peak response, and (C) recovery (see Experimental procedures).

DA treatment

Growth medium was removed and replaced with fresh growth medium containing 0 or 1 mM DA. The cells were exposed to DA for 4 h, and DA solution was changed once each hour over a period of 4 h. Following these incubations the cells were evaluated for alterations in Ca^{2+} flux. For determinations of Ca^{2+} flux, cells were washed three times with growth medium and loaded for 40 min with Fura 2-AM in DMEM containing 1% FBS. Fura-containing medium was removed, cells were incubated for 30 min in Kreb's Ringer's HEPES (KRH) buffer and tested immediately afterwards.

Ca^{2+} imaging

Ca^{2+} image analysis was performed as previously described (Leslie *et al*., 1985; Cheng *et al*., 1994). Briefly, the transfected cells were treated under one of the experimental conditions, washed with fresh media, and loaded with Fura-2/acetoxymethyl ester (2 μM) in loading medium (99% DMEM, 1% FBS) for 40 min at 37 °C with 5% CO_2, followed by a 30-min incubation in Kreb's-Ringer buffer (KRB: 1.3 mM $CaCl_2$; 131 mM NaCl; 1.3 mM $MgSO_4$; 5.0 mM KCl; 0.4 mM KH_2PO; 6.0 mM glucose; 20 mM HEPES; pH 7.4). A coverslip with treated COS-7 cells was inserted into a Leiden cover slip dish and 0.9 mL of KRH buffer added. This was placed into a Medical Systems Corp. open-perfusion microincubator with temperature control, which was mounted on the stage of an Olympus IMT-2 microscope and illuminated with a fluorescent light source. All tests were carried out at 37 °C.

Simultaneous images of cells at λ_{ex} 340/380 nm and λ_{em} 510 nm were captured using Simple PCI, a software package designed by Compix (Mars, PA, USA), to control a MAC 2000 filter/shutter controller (Ludl Electronic Products, Hawthorne, NY, USA). Pixel-by-pixel comparisons of the captured images were carried out and a ratio of Ca^{2+}-bound Fura (340 nm excitation wavelength) to unbound Fura (380 nm excitation wavelength) was generated for each pair of images. $[Ca^{2+}]_i$ was determined using the method of Grynkiewicz *et al*. (1985). The interval between capture ranged from 1.0 to 1.5 s. After approximately 45 s, the cells were depolarized by the addition

of 750 μM oxotremorine in the presence of 30 mM KCl, and image capture continued for an additional 6 min.

Measured parameters of $[Ca^{2+}]_i$

The following parameters were analysed as described in Joseph *et al*. (2002) and are shown in Fig. 6: (a) baseline: the average $[Ca^{2+}]_i$ seen before oxotremorine-induced depolarization; (b) increase: the proportion of cells showing increases in $[Ca^{2+}]_i$ in response to depolarization by oxotremorine of > 30% over baseline – only those cells that showed this magnitude of response were considered for further analysis; (c) recovery: a dichotomous variable calculated as the proportion (%) of cells in which $[Ca^{2+}]_i$ returned to 20% of the increase induced by oxytremorine within 300 s following depolarization.

Data analysis

Baseline $[Ca^{2+}]_i$ and peak depolarization $[Ca^{2+}]_i$ increases were analysed by analyses of variance (degrees of freedom are given in parentheses following the *F* value) using Systat (SPSS, Inc., Chicago, IL, USA) and *post hoc* Tukey's HSD multiple comparisons. Because response and recovery were dichotomous variables, data were analysed by Kruskal-Wallis one-way analyses of variance and Mann-Whitney *U*-tests. Cells that were counted as responders exhibited a ≥ 30% increase in depolarization following oxotremorine stimulation. Of the cells that responded, those cells that were able to reduce $[Ca^{2+}]_i$ within 300 s were counted as recovered and the percentage of the cells showing recovery for each condition was computed.

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